

Short Synthetic Terminators for Assembly of Transcription Units *in vitro* and Stable Chromosomal Integration in Yeast *S. cerevisiae*

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Abstract

Assembly of synthetic genetic circuits is central to synthetic biology. Yeast *S. cerevisiae* in particular has proven to be an ideal chassis for synthetic genome assemblies by exploiting its efficient homologous recombination. However, this property of efficient homologous recombination poses a problem for multigene assemblies in yeast since repeated usage of standard parts, such as transcriptional terminators, can lead to rearrangements of the repeats in assembled DNA constructs *in vivo*. To address this issue in developing a library of orthogonal genetic components for yeast, we designed a set of short synthetic terminators based on a consensus sequence with random linkers to avoid repetitive sequences. We constructed a series of expression vectors with these synthetic terminators for efficient assembly of synthetic genes using Gateway recombination reactions. We also constructed two BAC (bacterial artificial chromosome)

vectors for assembling multiple transcription units with the synthetic terminators *in vitro* and their integration in the yeast genome. The tandem array of synthetic genes integrated in the genome by this method is highly stable because there are little homologous segments in the synthetic constructs. Using this system of assembly and genomic integration of transcription units, we tested the synthetic terminators and their influence on the proximal transcription units. Although all the synthetic terminators have the common consensus with the identical length, they showed different activities and impacts on the neighboring transcription units.

Keywords

gene assembly, yeast, *Saccharomyces cerevisiae*, transcriptional terminator, bacterial artificial chromosome

Introduction

Recent progress in synthetic biology has made it possible to design and assemble biological parts into pathways or circuits of desired functions. The development of DNA assembly methods, including Golden Gate (1) and isothermal Gibson assembly (2), has led to the development of a number of efficient tools and strategies for assembly (3–13). These resources facilitate the construction of complex synthetic genetic circuits for different applications, for example, the production of biofuels and chemicals (14).

The yeast *Saccharomyces cerevisiae* is an attractive platform for such synthetic biology applications because it is a versatile and robust system for genetic manipulation and industrial biotechnology (14). One of the advantages of *S. cerevisiae* is its efficient homologous recombination *in vivo*. Since the initial application for plasmid constructions (15), yeast *in vivo* recombination has been widely exploited for precise DNA assemblies and genome manipulations. The assemblies of the first synthetic bacterial (16, 17) and eukaryotic (yeast)

chromosomes (18) highlight the value of this genome engineering tool. However, it poses a unique problem in synthetic circuit construction in yeast when multiple copies of a single standard biological part, such as transcriptional terminators, are present in tandem in the circuit, which may induce undesired recombination.

Eukaryotic terminator sequences are located in the 3' untranslated region, which define the position of transcription termination and poly(A) addition. Recent studies showed that expression of heterologous genes in yeast can be influenced significantly by transcriptional terminators; they demonstrated that the activities of terminators directly correlated with the stability of mRNAs and the expression level of the genes (19–21). Transcription terminators are therefore an important part for tuning gene expression when designing synthetic gene networks. Although the relative contribution of terminators in tuning synthetic gene expression may be small compared to those of promoters, combination of terminators with appropriate promoters has proved to be more effective strategy to improve gene expression, for example, in metabolic pathway engineering (19). Yet, the number of terminators in yeast synthetic biology toolbox still remains small (22).

In this study, we designed and characterized short synthetic terminators based on the consensus sequence proposed by Guo and Sherman (23, 24). Although a number of native yeast terminators are available for heterologous gene expression in yeast, synthetic terminators have advantages over native ones, as discussed by Curran *et al.* (20). First, synthetic terminators can be significantly shorter than native ones with minimal consensus elements. Second, short terminators would be ideal as standardized parts which, unlike native ones, have no unidentified functional sequence elements. Finally, synthetic terminators have little homology to the genome sequence therefore mitigating the risk of undesired recombination events, which may be an issue when assembling medium to large synthetic circuits or pathways. Curran *et al.* designed and characterized 30 synthetic terminators of varying lengths (20). Many of these terminators, however, share stretches of sequence homology, which may mediate homologous recombination in yeast if they are used in a tandem multigene assembly.

To avoid such repeated sequences, we have taken a different strategy for designing synthetic terminators, whereby short random sequences are used as linkers between the consensus sequences. These terminators thus have identical structure and length yet share minimum homology to each other.

We also designed and constructed a series of yeast expression vectors with the synthetic terminators for efficient assembly of transcription units (TUs) using Gateway recombination reactions. Two BAC (bacterial artificial chromosome) vectors were also constructed for assembling multiple TUs with the synthetic terminators *in vitro* and their integration in the yeast genome. The integrated tandem array of TUs in the genome thus created is highly stable because there are little homologous segments in the synthetic constructs. Using these plasmid vectors, we characterized the synthetic terminators and their influence on the expression of neighboring genes. We found that, although all the synthetic terminators are based on the same consensus sequence with an identical length, they have different activities and influences on the expression of adjacent genes.

Results and discussion

Design of short synthetic terminators

We designed and created ten new Gateway destination vectors for synthetic gene assembly, each of which has a unique short synthetic terminator (Figure 1). These vectors are similar to those we reported previously (25), harboring a yeast centromere element (*CEN6*), an origin of replication (*ARSH4*), a marker gene (*URA3*) and a Gateway recombination cassette (attR2-ccdB-Cm^R-attR1). According to Guo and Sherman (23, 24), yeast terminator signals consist of three elements; the efficiency element, the positioning element and the poly(A) site. We designed a set of synthetic terminators based on these consensus elements with random sequence linkers. The random linkers ensure no long stretch of homology (>25 bp) exists within the assembled DNA constructs so that the chance of undesired homologous

recombination is negligible. It was reported that *URA3* marker gene excision from the yeast genome failed using 25 bp direct repeat surrounding the gene (26), suggesting the efficient excision (deleterious recombination) of a DNA fragment requires repeated flanking sequences longer than 25 bp. Unlike those reported by Curran *et al.* (20), these terminators have identical structure and length with minimum homology to each other.

To create the random sequence linkers, we employed the R2oDNA Designer (4, 27) to design short random sequences of 10 bp long that are biologically neutral in both *E.coli* and *S. cerevisiae*. R2oDNA Designer can exclude inappropriate sequences from the random sequences, for instance, restriction enzyme sequences, transcription factor binding sites, poly(A) runs or sequences homologous to the host (*i.e.* yeast) genome (4, 27). These random sequences link the three consensus terminator elements (23, 24) (Figure 1). Note that one of the synthetic terminators, T0, is identical to the one reported previously (20, 23). Random sequences also link the Gateway recombination cassette and the synthetic terminator to the vector backbone (Figures 1 and 2).

A synthetic gene can be assembled on any of these vectors efficiently by a single Multisite Gateway recombination reaction *in vitro* with the Gateway entry clones of a promoter and an open reading frame (ORF) (Figure 2a) (25). The assembled gene (transcription unit; TU) is flanked by Prefix and Suffix (15bp) primer binding sequences (adopted from Casini *et al.* (4)) for the amplification of the TU by PCR. Note that attB2, attB5 and attB1 sites (21 bp each) left behind after the Gateway recombination reaction do not interfere with the expression of the gene (25). The expression of assembled genes with synthetic terminators can be tested by simply transforming yeast with the vectors. This allows verification of the functions of synthetic TUs before they are assembled together and integrated in the yeast genome as described later. This is one of the unique advantages of our system.

Activities of synthetic terminators evaluated by fluorescent protein expression

To evaluate the activities of the synthetic terminators, we measured the expression of the fluorescent proteins yVenus (yeast-optimized Venus fused to the destabilizing degenon Cln2_{PEST}(28) and a nuclear localization signal (NLS), which we simply call yVenus herein) and mCherry-NLS (which is called mCherry herein) under the control of a constitutive promoter (strong *S. cerevisiae* *TEF* (25) or medium-strength *Schizosaccharomyces pombe* *adh1* promoter, which we refer to as *ADH1* herein) by flow cytometry (Figure 3) and Supplementary Figure S1). Note that cloning of some constructs (the Gateway destination vector with the synthetic T7 terminator and *TEF*-yVenus-T6 construct) failed with an unknown reason despite our repeated attempts. Despite the fact that the terminator sequences are similar with the same GC content ($\sim 40\%$) for random linkers, they confer different levels of fluorescent protein expression over several folds. T0 terminator gave the highest fluorescence while T1 terminator had the lowest activity among the terminators tested. Other terminators resulted in intermediate levels of fluorescence. The terminator sequence T0, which was originally described by Guo and Sherman (23, 24), has a comparable level of activity to the commonly used *TEF* terminator (from *Ashbya gossypii*) (Figure 3). Curran *et al.* reported that the expression of a reporter gene with T0 (T_{Guo1} in their notation) terminator conferred 2.3 fold higher expression than that with the CYC1 terminator, another commonly used terminator in yeast (20). Extrapolating their results to ours, the synthetic terminators with intermediate level of activities (T2, T3, T4, T6, T8, T9, T10) can confer expression efficiencies similar or higher than that of CYC1.

Curran *et al.* also extensively examined the influence of synthetic terminator sequence on the mRNA’s structure and stability (20). They found several design principles for efficient gene expression including the spacing between the consensus elements and GC content. They also found a strong correlation between the transcript and the expression levels. The reason for the different activities among our synthetic terminators, which are similar to each other,

is unknown, but the difference might be at the transcript level.

Design and construction of BAC vectors for the assembly of transcription units and genomic integration

To assemble multiple TUs and integrate them together in the yeast genome, we constructed a couple of new BAC (bacterial artificial chromosome) vectors (Figure 2b), that allow stable integration of a DNA construct by homologous recombination into the chromosomal loci (25, 29, 30). We adopted an approach for multiple gene assembly modified from those reported previously (4, 7, 12, 31) (unique nucleotide sequence (UNS)-guided assembly, i.e., an overlap-directed DNA assembly). UNSs are 40bp random nucleotide sequences and were also designed by the R2oDNA Designer (4, 27). The BAC integration vectors contain UNSs, U1 and Ux, which link TUs to the vector in an isothermal assembly *in vitro*. The vectors also harbor selection markers chloramphenicol-resistance Cm^{R} and the yeast *URA3* genes.

TUs assembled by Gateway recombination reactions can be amplified by PCR (Figure 2b, c) using primers with a pair of Prefix/Suffix linked to appropriate UNS overhang sequences (UNS primers; see Supplementary Table 1, "PCR primers for gene assembly" for sequences). Each TU amplified by PCR harbors overlapping UNS sequences that link TUs together by isothermal (Gibson) assembly reaction (Figure 2c, d). Figure 2e-f depicts the assembly and integration events of three TUs at *HIS3* locus as an example. The U1-Ux site in the vector is flanked by YIp-In/Out sequences; YIp-In mediates integration of the assembled TUs in the genome (either at *HIS3* or *FCY1* locus, Figure 2e); YIp-Out mediates the removal of the vector backbone and the genomic marker gene by homologous recombination (Figure 2f). This integration and removal by homologous recombination leaves behind no duplicated growth marker gene segments, making the integrated construct stable in the genome.

Transcriptional interference in tandem synthetic TUs

Using the method described above, we assembled two TUs encoding fluorescent protein reporters (yVenus and mCherry) with the synthetic terminators and integrated them in the genome (Figure 4a). The upstream TU consists of *TEF* promoter, yVenus ORF and a synthetic terminator; the downstream TU contains the tetracycline-inducible promoter (*TetO7-CYC1TATA*)(25), mCherry ORF and the *TEF* terminator. Fluorescence intensities of yVenus and mCherry in the presence or absence of the inducer doxycycline (Dox) were measured by flow cytometry (Figure 4b and Supplementary Figure S2). In the absence of Dox, in all strains, yVenus was expressed but not mCherry. The relative expression (fluorescence) levels of yVenus with different terminators in the genome correlate well with those from the plasmid constructs, especially with those of *ADH1*-mCherry (Figure 5). This is one of the advantages of our gene assembly strategy since each TU can be tested individually on a plasmid before being assembled together with other TUs.

Unexpectedly, in some constructs upon induction by Dox, yVenus expression was affected when the downstream mCherry was induced (Figure 4b); those with T2 and T3 terminators exhibited a marked decrease while those with T4, T9 and T10 showed a significant increase in yVenus expression. The expression of yVenus also affected that of mCherry downstream, which was evident in the two constructs with T0 terminators; the only difference between them is the promoters (minimal *HIS3TATA* or strong *TEF* promoter) (Figure 4b, first four rows). The mCherry fluorescence was significantly lower when the upstream yVenus was highly expressed by *TEF* promoter. A similar interference was observed in a different construct with three TUs in tandem (Figure 6). Both the 2-gene and 3-gene constructs have the identical yVenus TU with the constitutive promoter *TEF* (Fig. 6a, b). Note that the 2-gene construct has the TU for rtTA at a separate genomic locus. In the 3-gene construct, induction of mCherry (with T9 terminator) by Dox reduced the expression of the downstream yVenus TU by almost three fold, whereas in the 2-gene construct, induction of mCherry (with *TEF* terminator) by Dox resulted in a modest reduction of yVenus expression. Despite the fact

that all synthetic terminators tested here were designed based on a consensus sequence (Figure 1), they showed different activities and influence on adjacent TUs' expression. Together, these results indicate the expression level of tandem compact TUs are influenced by a local context such as promoters and terminator sequences in yeast *S. cerevisiae*.

What is the mechanism of this transcriptional interference? Transcription of antisense ncRNAs is one possible mechanism. Recent studies revealed that in yeast hundreds of noncoding RNAs (ncRNAs) are made by pervasive bidirectional transcription by RNA polymerase II (Pol II). ncRNAs that are antisense to the coding mRNA may interfere with the expression of the gene. Several mechanisms have been shown to restrict pervasive ncRNA transcription in yeast: 1) gene looping, which brings the promoter and terminator together and enhances directionality of transcription (32); 2) chromatin remodeling by deacetylation (33); 3) RNA degradation by the exosome (34, 35); 4) Nrd1/Nab3-dependent selective termination of ncRNA transcription (36–38). The synthetic tandem array of TUs may lack the sequence elements or chromatin structure essential for some of the above mechanisms, leading to ncRNA transcriptions that may interfere with the expression of TUs. Read-through transcription is another possible mechanism of the interference. Incomplete termination and transcriptional read-through may cause transcriptional interference of the TU immediately downstream. Our results showed that the tetracycline-responsive promoter (*TetO7-CYC1TATA*) can interfere with the *upstream* TU's expression when the promoter is active (i.e., in the presence of Dox).

Another possible explanation for the transcriptional interference might be a metabolic burden imposed by heterologous gene expression, such as that of fluorescent proteins. The main cause of the metabolic burden is thought to be a depletion of resources required for gene expression, for instance, the free ribosome pool (39). In this scenario, an induction of a fluorescent protein is expected to reduce the expression of other genes; however, that was not always the case in the tandem TU arrays tested in this study, for example, the expression of yVenus with T4 and T10 terminators increased after the mCherry induction by Dox (Fig.

4b).

Alternatively, gene looping may mediate the transcriptional interference since it is induced by the activation of a promoter (40). In a tandem array of compact TUs like the ones tested in this study, each terminator (except the last one) is immediately followed by the promoter of the next TU. Gene looping in the tandem configuration of the TUs may juxtapose multiple promoter and terminator regions physically, thereby causing a competition for the local pool of Pol II among TUs and transcriptional interference. Stringent termination might be achieved by introducing the binding motifs of Nrd1 and Nab3, which bind cooperatively to RNA (41). Casini *et al.* suggested that synthetic linker sequences may provide a necessary space between TUs for insulation against transcriptional interference (4). It would be interesting to examine how the length of intergenic region affects the local gene expression in yeast and find the minimum length of UNS for efficient intergenic insulation.

Alternatively, the influence of terminators on the proximal TU's expression can be exploited as demonstrated in the recent study (42), which utilized terminators as components for genetic band pass filter in *E. coli*. This study has indicated that it may also be possible in eukaryotic cells to use terminators as sophisticated but simple control elements for gene expression in synthetic gene circuits.

Predicting the degree of transcriptional interference by terminators, however, may not be trivial. We examined the chromatin occupancy of DNA sequences using a computational model (a duration hidden Markov model (43)). Curran *et al.* exploited this model successfully to design synthetic yeast promoters (44). However, we found no obvious correlation between the predicted nucleosome occupancy around the synthetic terminator and the expression profile of the gene constructs tested (data not shown). It seems that transcriptional interference with a synthetic terminator is context-dependent.

Together, this study demonstrates a valid and useful strategy for developing a library of orthogonal synthetic terminators with little homologous sequences to avoid unwanted homologous recombination in yeast. These synthetic terminators are significantly shorter than

native ones thus help reduce the size of assembled DNA constructs. Although integration by homologous recombination in yeast is highly efficient, our method may have limitations on the size of the DNA construct that can be integrated in the genome efficiently. To overcome this possible technical bottleneck, our system of gene assembly and chromosome integration can be improved in the future by combining it with a homing endonuclease(45, 46) or the clustered regularly interspaced short palindromic repeats-associated (CRISPR-Cas) system (46–49). We have also showed that although all the synthetic terminators are based on the same consensus sequence, these synthetic terminators have different activities and influences on the expression of adjacent genes. Further experimental and theoretical studies will be necessary for predicting the transcriptional interference from the DNA sequence information alone, which would aid the design and construction of synthetic genetic circuits by assembling TUs.

Methods

Strains and media

Saccharomyces cerevisiae strains used in this study are listed in Table 5, which are all derived from S288C. Yeast cells were grown at 30 °C in YPD rich medium or SC medium lacking appropriate amino acids (50). For genetic manipulation and transformation of yeast, standard protocols were used as previously described (25, 50). Yeast cells were grown until the culture reached early to mid-log phase ($OD_{600} = 0.1 \sim 1.0$) for flow cytometry. BAC plasmid vectors were maintained and propagated in NEB10 β (New England Biolabs) *E. coli* strain, according to the manual of pETcocoTM system (Novagen). To maintain the BAC vectors in the single copy state, *E. coli* cell cultures were cultured in LB medium containing 0.2 % glucose and 12.5 μ g/ml chloramphenicol. To amplify the copy number of a BAC vector for DNA purification, cells transformed with the plasmid were cultured in LB+glucose (0.2 %)+chloramphenicol (12.5 μ g/ml) overnight at 37 °C; the overnight culture was diluted

1:50 into LB+chloramphenicol (12.5 $\mu\text{g/ml}$) without glucose and culture at 37 °C until $\text{OD}_{600} = 0.2 \sim 0.4$; then L-arabinose was added to the culture to 0.01 %; cells were cultured further for 4 to 5 hours at 37 °C before harvesting.

Plasmid construction

PCR reactions were performed using Q5[®] high-fidelity DNA polymerase (New England Biolabs) according to the manufacturer’s instructions. The composition of typical reactions was 1 \times Q5 reaction buffer with 50 μM each dNTP, 0.5 μM each primers and 5 ng of template DNA in a 50 μL reaction. PCR conditions and primers are summarized in Supplementary Table 2. PCR fragments were purified by QIAquick PCR Purification Kit (Qiagen) before further enzymatic reactions. The Gateway recombination reactions were performed using BP clonase[®]II or LR clonase[®]II enzyme mix (Thermo Fisher Scientific) according to the manufacturer’s manual. NEBuilder[®] HiFi Assembly Master Mix (New England Biolabs) was used for isothermal (Gibson) DNA assembly reactions. rAPid alkaline phosphatase (Roche) was used to treat linear DNA vectors prior to ligation reactions. DNA ligation was performed with Rapid DNA Ligation kit (Roche). Restriction enzymes were obtained from New England Biolabs or Promega.

Each Gateway destination vector with a synthetic terminator was assembled from three PCR fragments (Gateway cassette, T7 terminator, Synthetic terminator in Supplementary Table 2) and the vector backbone pDEST416TEFt7 (25) digested by SacI and KpnI prior to the assembly. The BAC vectors, pDEST373-U1Ux and pDEST374-U1Ux, were constructed as follows. pETcoco-1 (Novagen) was linearized by PciI and AvrII. The 9.9 kb vector backbone was purified by gel electrophoresis and QIAquick gel extraction kit (Qiagen). U1Ux PCR fragment (Supplementary Table 2) was digested by PciI and NheI and ligated to the purified pETcoco-1 vector backbone, creating the plasmid pU1Ux. pU1Ux was linearized by BstZ17I, dephosphorylated and purified by QIAquick PCR Purification Kit. The linearized pU1Ux was subjected to an isothermal DNA assembly with *URA3* PCR fragments

(Supplementary Table 2), which created the plasmid pDEST-U1Ux. *FCY1* YIp-Out PCR fragments (Supplementary Table 2) were digested with EagI and ligated to the linearized pDEST-U1Ux (with NotI) to create pYS76. pYS76 was then cut by PspXI and ligated to *FCY1* YIp-In PCR fragments (Supplementary Table 2) digested with XhoI. This resulted in the BAC vector pDEST373-U1Ux. Similarly, *HIS3* YIp-Out fragments (Supplementary Table 2) were treated with XhoI and ligated with the linearized pDEST-U1Ux (by PspXI), which resulted in the plasmid pMM55. pMM55 was then cut by NotI and ligated to the *HIS3* YIp-In PCR fragment (Supplementary Table 2) which was pre-treated with EagI. This created the BAC vector pDEST374-U1Ux. GenBank accession numbers for pDEST373-U1Ux and pDEST374-U1Ux are KX344514 and KX344515, respectively.

HIS3 TATA minimal promoter was amplified from pRS413 (51) by PCR to make the Gateway Entry clone pDV19 after several steps of subcloning. A partial sequence of the plasmid between Gateway recombination sites (attL2 and attL5) is provided in Supporting Information. All new plasmid vectors were verified by DNA sequencing.

Design of random linkers and UNSs

R2oDNA Designer (4, 27) was used to design random linkers and UNSs. See Supplementary Table 3 for query sequences. Recognition sequences of four restriction enzymes (NruI, MscI, KpnI and SacI) were excluded in addition to the default setting for the random sequence generation.

Flow cytometry

Overnight precultures were diluted to early log phase ($OD_{600} = 0.2$) and incubated for six hours in the presence or absence of 1 μ g/ml doxycycline. Flow cytometry was then performed as described previously (25) recording 10,000 cells per sample. Raw gated data was exported as csv files and plotted in Matlab.

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Supporting Information Available

Supplementary Tables 1 to 5 and figures S1 and S2 (oligonucleotide sequences, PCR condition, plasmids used in this study and the flow cytometry data corresponding to Figure 3 and 4).

This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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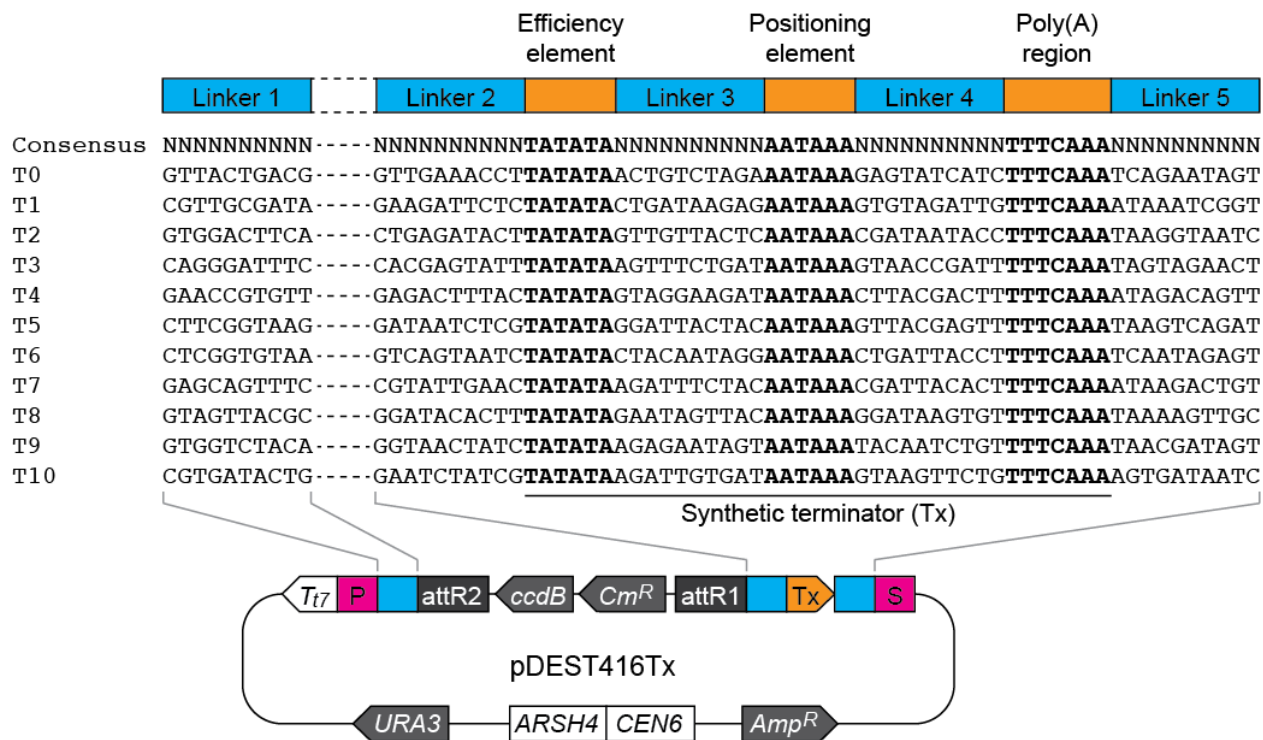


Figure 1: Yeast Gateway destination vector for gene assembly with synthetic terminators. Schematic diagram of the vector (pDEST416Tx; Tx stands for synthetic terminators T0, T1, ..., T10) is shown with the terminator sequences. Each vector harbors sequence elements including: replication origin ARSH6, centromere element *CEN6* and yeast *URA3* marker for plasmid maintenance in yeast and Gateway recombination cassette (attR2- *ccdB*-*Cm^R*-attR1). Abbreviations: Amp^R, ampicillin resistance gene; Cm^R, chloramphenicol resistance gene; Tt7, T7 terminator; P, Prefix; S, Suffix sequence.

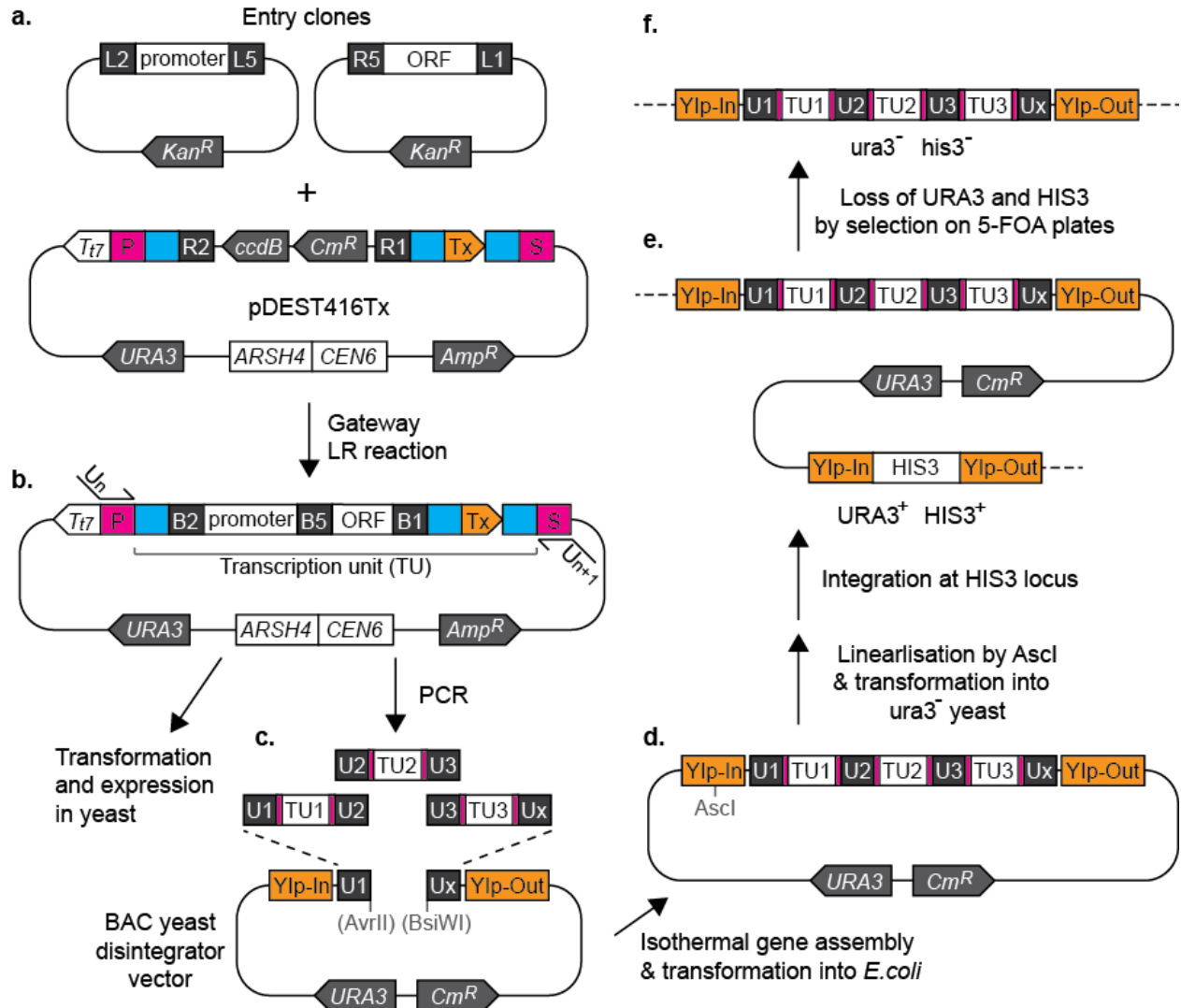


Figure 2: Assembly and genomic integration of transcription units. (a, b) Assembly of a synthetic transcription unit (TU) by multisite Gateway recombination reaction. A single TU is assembled from two Entry clones and a destination vector pDEST416Tx (a), resulting in a yeast expression vector (b). Each TU is flanked by Prefix (P) and Suffix (S) primer binding sequences. (c) TUs are amplified by PCR using primers with an overhang of unique nucleotide sequences (UNS; U_n , $n = 1, 2, \dots$ and U_x). Multiple TUs are assembled onto the BAC vector by UNS-guided isothermal reaction (d). The resulting vector is then linealized by *AscI* enzyme and transformed into yeast. (e) Integration is mediated by homologous recombination between YIp-In and the target genomic locus (*HIS3* in this example). (f) YIp-Out (also homologous to the *HIS3* target locus) mediates the removal of the vector backbone and the genomic marker gene (*HIS3*) by homologous recombination.

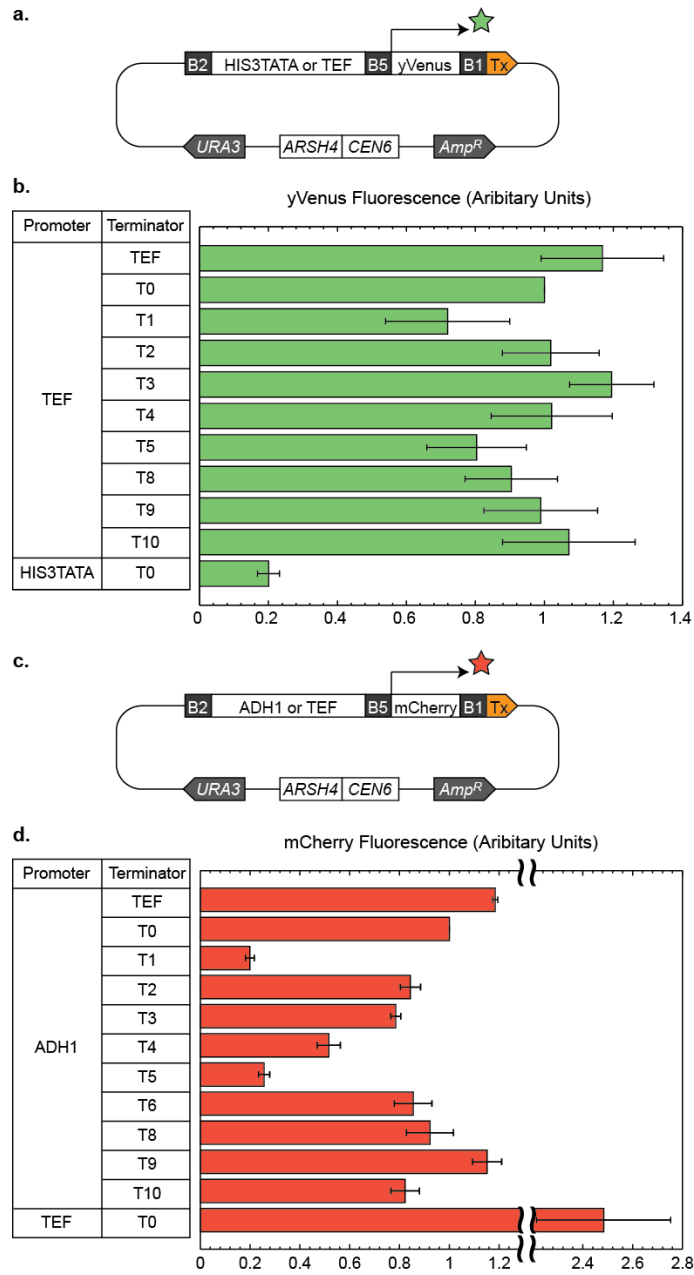


Figure 3: Expression of the synthetic transcription units (TUs) assembled by multisite Gateway recombination reaction. (a) Illustration of the assembled plasmids that constitutively express the yellow fluorescent protein yVenus. (b) The plasmids from (a) were transformed into yeast and fluorescence recorded by flow cytometry. Data represents the mean fluorescence intensity with error bars (Standard Error of the Mean; SEM) from three independent experiments. (c) Depicts the assembled plasmids that constitutively express the red fluorescent protein mCherry. (d) mCherry plasmids were transformed in yeast and recorded as per (b).

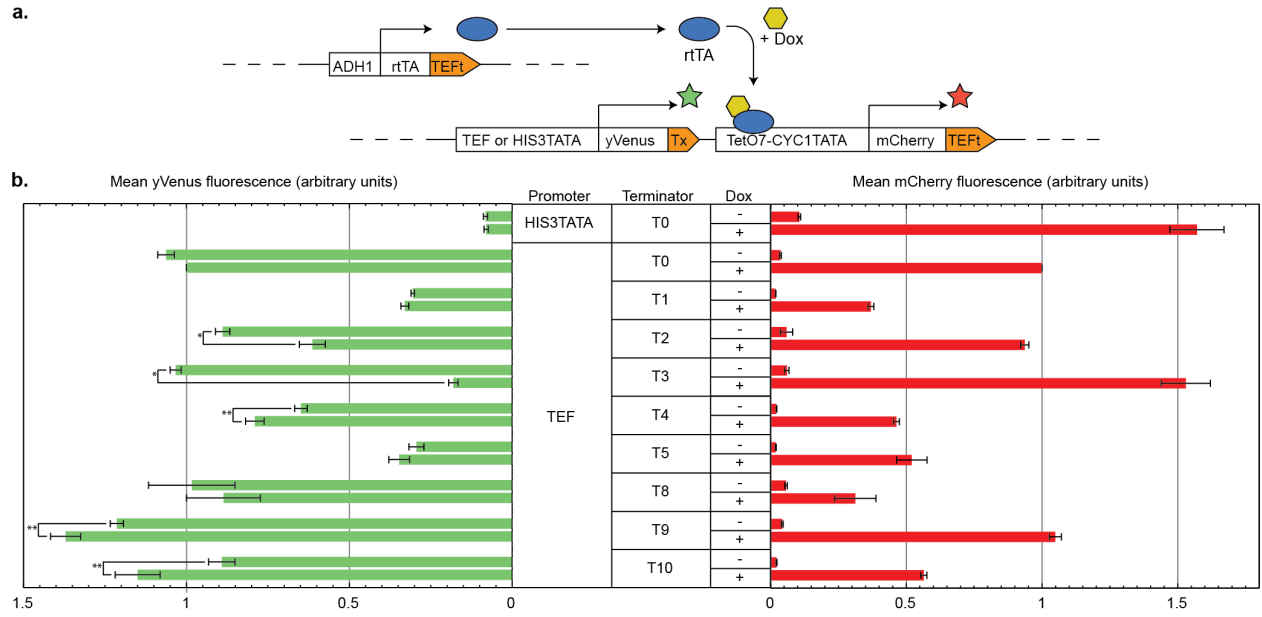


Figure 4: Influence of synthetic terminators on adjacent TU's expression. (a) Cartoon showing two TUs integrated into yeast where the upstream gene contains a synthetic terminator (Tx). (b) Strains were grown in the presence or absence of doxycycline (Dox) and the fluorescence was recorded by flow cytometry. Mean fluorescence intensity \pm SEM is shown from three independent experiments. Asterisks * or ** indicate samples showing a significant decrease or increase in expression, respectively, by the addition of Dox (*, $p \leq 0.005$; **, $p \leq 0.05$).

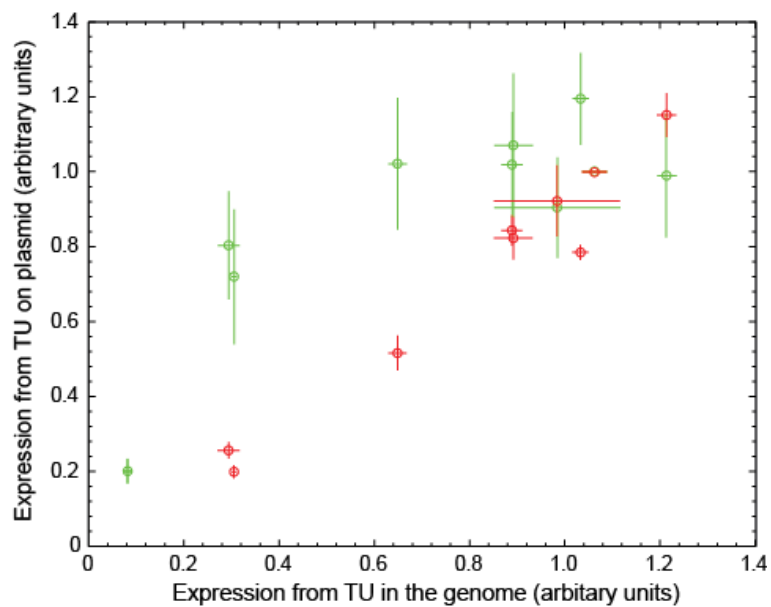


Figure 5: Correlation of gene expression from TUs expressed on plasmids to those integrated in the genome. Mean fluorescence intensity \pm SEM from triplicate experiments (the same data sets as in Fig. 3 and 4) are shown. The scattered plot shows the expression from the integrated yVenus TUs (*HIS3TATA*- or *TEF*-yVenus in the absence of Dox) against the same TUs on plasmids (green) or against mCherry TUs on plasmids (*ADH1*-mCherry with the same synthetic terminators; red).

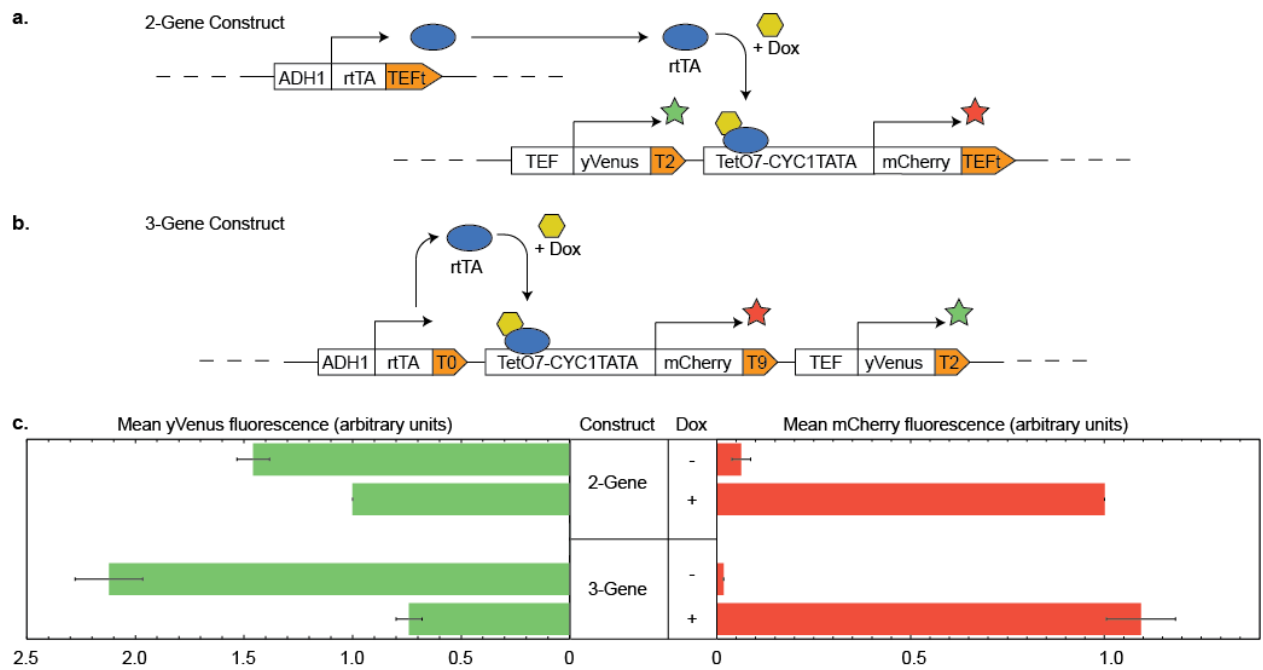


Figure 6: Expression of multiple TUs in different contexts in the yeast genome. (a) Schematic diagram showing two TUs integrated together at the *FCY1* genomic locus. (b) Illustrates three TUs that were integrated at the *HIS3* locus. (c) Yeast strains were grown in the presence or absence of Dox and fluorescence measured by flow cytometry. Graph depicts the mean fluorescence intensity \pm SEM from three independent experiments. Note that this experiment was done simultaneously with the data acquisition for Fig. 4. The two-gene construct represents the same data shown for T2 in Fig. 4.